How to obtain a rhodopsin spectrum from a turbid suspension - Beating the light scatter problem

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PURPOSE AND BACKGROUND:

Our goal is to develop assays to monitor retinoid delivery/removal in photoreceptor suspensions and intact retinas. This will facilitate studies of the structure & function of interphotoreceptor retinoid-binding protein (IRBP) in the normal rod and cone visual cycles **(Fig 1a**), and pathological states such as retinitis pigmentosa **(Fig 1b)**. However, spectroscopy of cellular suspensions is severely limited by light scattering **(Fig 2)**. Here we report the use of integrating cavity absorption meter to obtain reliable rhodopsin spectra from turbid outer segments suspensions. We have now extended these studies to dispersed and intact living retina.

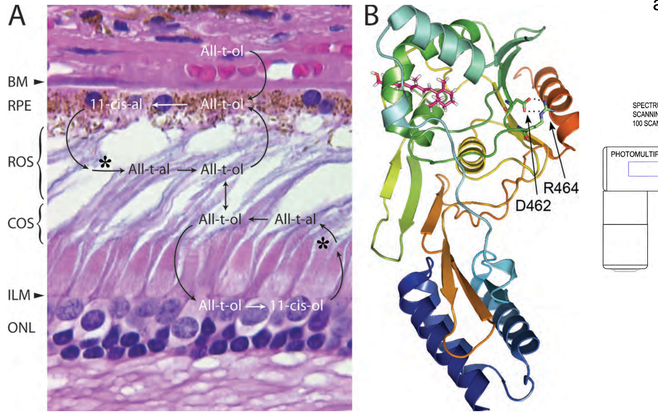

Fig 1. Visual cycle, and structure of IRBP. A) 11-*cis* retinal (11-cis-al) is photoisomerized (*) to its 11-*cis* isomer that is reduced to all-*trans* retinol (All-t-ol). The All-t-ol is then released from the rod and cone outer segments into the interphotoreceptor matrix. From Gonzalez-Fernandez (2012)[1]. **B)** Module II *Xenopus* IRBP docked with all-*trans* retinol. A salt bridge extends between the carboxamide side group of D462, and guanidinium group of R464. The corresponding aspartic acid of human IRBP is replaced by asparagine in a form of retinitis pigmentosa. Adapted from Hollander *et al.* (2009)[2]. IRBP is thought to facilitate retinoid trafficking by protecting retinoids from photodegradation while targeting their delivery and uptake. The methods described here will provide new assays to study the mechanism of IRBP trafficking in the visual cycle

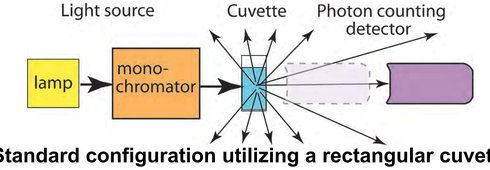

Fig 2. Standard configuration utilizing a rectangular cuvette. Light scatter limits the usefulness of this arrangement for cell and tissue suspensions. Rhodopsin spectra from turbid suspensions have been obtained by moving the detector closer to the cuvette compartment (detector outlined with dashed lines)[3, 4]. Nevertheless, light scatter remains a significant problem, and most physiological assays of IRBP function in living cells have resorted to single cell microspectroscopy[5].

METHODS:

Outer segments were prepared by shaking dark-adapted bovine retina in PBS. Spectra were obtained using: 1) A rectangular cuvette in a HP diode array spectrophotometer, and 2) An 8 ml spherical cuvette surrounded by a tightly packed proprietary white powder serving to maximize reflectance of light on the interior walls of the flask **(Figs 3,4).** Light was delivered using an Olis RSM 1000 UV/Vis rapid-scanning spectrophotometer **(Fig 3)**. The apertures to the sphere through which the measuring light entered and the transmitted/scattered light exited to the photomultiplier tube were at 90° angles. The absorbance / cm was calculated using algorithms described by Javorfi et al (2006). [6] We have now extended these studies to dispersed and intact frog (*Rana pipiens*) retina suspended in Ringer's **(Figs 7-9).**

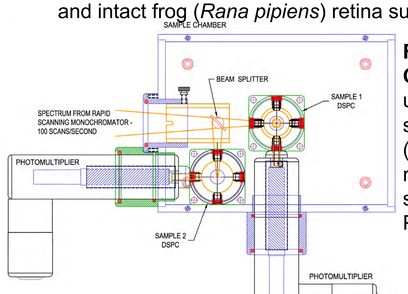

Fig 3. Diagram of the dual beam CLARITY spectrophotometer. This unit is equipped with an Olis rapid scaning UV / VIS mono-chrometer (RSM). The conventional single pass rectangular cuvette is replaced with a spherical cuvette, the DeSa Presentation Chamber (DSPC).

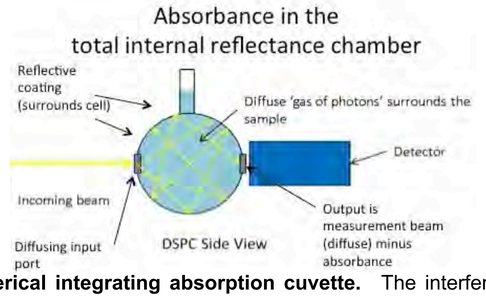

Fig 4. Spherical integrating absorption cuvette. The interference of light scattering is eliminated through the use of a spherical cuvette surrounded by a reflective material. The measurement beam is fully scattered (yellow lines). The effective path-length is markedly increased (tens of centimeters). DeSa Presentation Chamber (DSPC).

RESULTS:

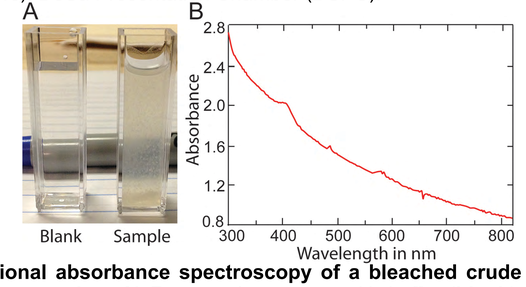

Fig 5. Conventional absorbance spectroscopy of a bleached crude outer segment suspension. A) Rectangular cuvette with buffer (**blank**); bleached outer segment preparation (**sample**). **B)** Absorbance spectrum obtained using a rectangular quartz cuvette in a HP 8452 diode array spectrophotometer. The absorbance is dominated by Rayleigh scattering.

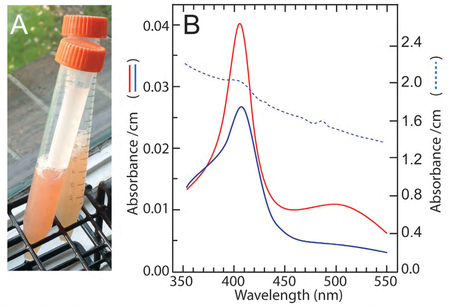

Fig 6. Rhodopsin spectra obtain using the integrating cavity absorption chamber. A) Crude outer segment suspension before (**left**) and after (**right**) bleaching. **B)** Absorbance spectra obtained before (**red**), and after (**blue**) bleaching under direct sunlight. Data from **Fig 5** is superimposed (dashed line).

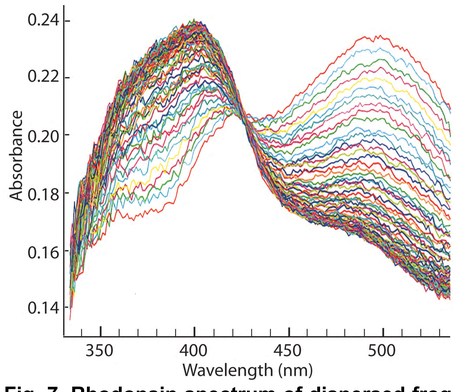

Fig. 7. Rhodopsin spectrum of dispersed frog retina within the integrating cavity absorption chamber. Scans: 40 sec at 2 scans/sec with exposure to 515 nm LED at 100 flashes/sec..

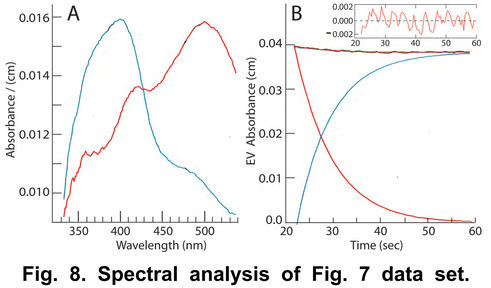

Fig. 8. Spectral analysis of Fig. 7 data set. Absorbance was corrected according to Javorfi et al (2006)[6]. **A**) **Red**, pre-bleach; **blue**, post-bleach spectra. **B**) Data was fit to two species: A -> B with rate constant of 0.132 sec^{-1}.

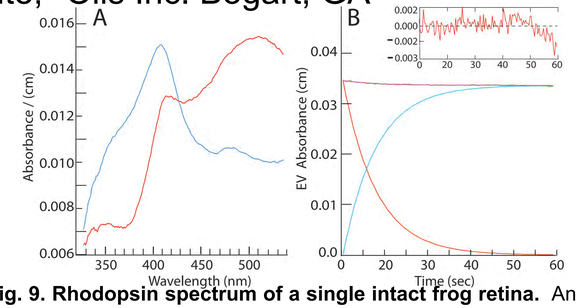

Fig. 9. Rhodopsin spectrum of a single intact frog retina. An intact neural retina was detached and transferred directly to the integrating cavity where it was exposed to 515 nm LED at 100 flashes/sec. Scans were taken for 40 sec at a rate of 2 scan/sec. Absorbance was corrected according to Javorfi et al (2006)[6] **A) Red**, pre-bleach; **blue**, post-bleach spectra. **B**) Data was fit: A -> B; rate = 0.105 sec^{-1}.

SUMMARY & REFERENCES:

The integrating absorption sphere is a reflecting cavity acting as a multi-pass cuvette. Fry et al (1992)[7] attributed the idea to Elterman (1970)[8], who "emphasized that, if a sample is in an isotropic homogeneous field, then the absorbed radiant power is independent of scattering effects." To our study is the first to use of this technology to vision biochemistry. Reliable spectra were obtained from turbid suspensions. Furthermore, we have extended this work to dispersed and intact living amphibian retina. The higher effective path-length enhances sensitivity, and can be accounted for allowing determination of the absorbance / cm[6, 9]. The overall approach will allow use of photoreceptor suspensions, and intact retina for new assays of the vitamin A cycle.

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